

Accumulation of AGEs and VEGF in Eyes of SDT Rats

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Abstract: *Background/Aims:* The Spontaneously Diabetic Torii (SDT) rat develops advanced diabetic retinopathy (DR). The aim of this study was to identify advanced glycation end products (AGEs) related to vascular endothelial growth factor (VEGF) expression, a cause of DR in SDT rats.

Methods: One eye was obtained from six SDT rats (blood glucose, >250 mg/dl) and 10 nondiabetic normal Sprague-Dawley (SD) rats and prepared for immunohistochemical study of VEGF and AGEs (pyrraline, pentosidine, carboxy methyl lysine [CML]). Immunostaining was described as minimal, moderate, and severe.

Results: In diabetic rats, for CML, five eyes had severe and one moderate immunostaining. For pyrraline, one eye had moderate and five eyes minimal immunostaining. For pentosidine, one eye had moderate and five eyes minimal immunostaining. For VEGF, three eyes each had moderate and severe immunostaining. In nondiabetic rats, for CML one eye had minimal, seven had moderate, and two had severe immunostaining. For pyrraline, four eyes had moderate and six eyes minimal immunostaining. For pentosidine, 10 eyes had minimal immunostaining. For VEGF, one eye had moderate and nine had minimal immunostaining. The prevalence rates of CML and VEGF were significantly ($P < 0.05$, $P < 0.001$, respectively) greater in diabetic than in nondiabetic rats. The prevalence rates of pyrraline and pentosidine were not significantly ($P = 0.35$, $P = 0.38$) different between diabetic and nondiabetic rats.

Conclusion: CML coexists with VEGF and may be involved in the pathogenesis of severe ocular complications in SDT rats.

Keywords: Diabetes, diabetic retinopathy, advanced glycation end products (AGEs), vascular endothelial growth factor (VEGF).

INTRODUCTION

Diabetic ocular complications, such as diabetic retinopathy (DR), cataracts, and rubeotic glaucoma, impair vision and quality of life. DR, one of the most serious complications of diabetes mellitus, frequently leads to blindness [1-3]. Vascular endothelial growth factor (VEGF), an important cytokine that induces proliferative DR [4,5], is correlated with several metabolic changes, including increased polyol pathway activity [6-9], activation of protein kinase C (PKC) [10-14], increased oxidative stress [13,15,16], and accumulation of advanced glycation end products (AGEs) [13,17-19]. These induce retinal vascular dysfunction and retinal ischemia. New drugs targeting these biochemical changes, such as aldose reductase inhibitors [6,20], PKC β inhibitors [11,12,14,21-23], and AGE inhibitors [24,25], are effective in diabetic animal models of very early DR, in which pericyte loss occurs. However, to

determine whether these drugs effectively prevent DR, an animal model of advanced DR is needed. Although numerous diabetic animal models have been described, none develops DR similar to that in humans.

The SDT rat, a substrain of the Sprague-Dawley (SD) rat, spontaneously develops diabetes mellitus and exhibits the three major diabetic ocular complications, cataracts, advanced DR, and rubeotic glaucoma. In 1988, five male rats with polyuria and glucosuria were identified among 305 rats from an outbred colony of the Crj:CD(SD) strain (Charles River Japan, Inc., Kanagawa, Japan) of SD rats. After the 20th generation of sister-brother matings, the diabetic strain was established in 1997. The characteristics of this rat have been described previously [26]. Briefly, male rats develop marked hyperglycemia (about 700 mg/dl) and glucosuria after 20 weeks of age. The cumulative incidence of diabetes is almost 100% by 40 weeks of age. Female rats also develop diabetes but after 45 weeks of age, and the cumulative incidence is only 35% even after 60 weeks of age. The survival rates of untreated male and female SDT rats up to 65 weeks are 93% and 97%, respectively. Mature diabetic cataracts are observed after 40 weeks of age in most male SDT rats. Large retinal folds mimicking tractional retinal detachment with extensive leakage of fluorescein around the

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optic disc are the most prominent finding of DR in old (mostly after 51 weeks of age) SDT rats [27]. Young SDT rats (under 50 weeks of age) usually do not exhibit the changes of advanced DR. In the current study, we performed immunohistochemical studies that focused on AGEs and VEGF in SDT rats.

ANIMALS AND METHODS

After 60 weeks of age, one eye was obtained from 10 nondiabetic normal SD rats and six SDT rats. SDT rats were confirmed to be diabetic (blood glucose level, >250 mg/dl). The care and handling of all animals were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Under deep anesthesia following an intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight, Nembutal, Dainihonsei-yaku, Osaka, Japan), the eyes were enucleated for conventional histopathologic and immunohistochemical studies. The eyes were fixed in a mixture of 2.5% paraformaldehyde and 1% glutaraldehyde in 0.15 M phosphate buffer (pH 7.1) to avoid artificial retinal detachment and embedded in paraffin and sectioned for immunohistochemical study of VEGF and AGEs (pyrraline, pentosidine, carboxy methyl lysine [CML]). The immunohistochemical procedures were based on the standard avidin-biotin horseradish peroxidase method using each antibody and developed with AEC Substrate Chromogen (DakoCytomation, Carpinteria, CA, USA). VEGF was immunostained with a monoclonal antibody for human VEGF (1:25 dilution, Immuno-Biological Laboratories Co., Ltd, Fujioka, Japan). Pyrraline, pentosidine, and CML also were immunostained with a monoclonal antibody for human AGEs (1:50 dilution for pyrraline, 1:50 dilution for pentosidine, and 1:50 dilution for CML, Trans Genic Inc., Kumamoto, Japan). Bovine serum was used as a primary antibody for negative control of the immunostaining. The immunostaining grades were divided into three groups, minimal, moderate, and severe, according to the degree of staining. Minimal staining was characterized by almost no retinal staining, moderate staining by light red retinal staining, and severe staining by strong dark red retinal staining. We evaluated the grade of the immunostaining in each sample without knowing whether the eye was obtained from a SD or SDT rat.

The prevalence rates of AGEs and VEGF were evaluated using the Cochran-Armitage test and Fisher's exact test for independence. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the prevalence rates of immunostaining for CML, pyrraline, pentosidine, and VEGF in the retinas of the diabetic rats. Fig. (1) shows immunostaining for CML, pyrraline, pentosidine, and VEGF in the retinas of SDT rats. Table 2 shows the prevalence rates of immunostaining for CML, pyrraline, pentosidine, and VEGF in the retinas of nondiabetic SD rats. Fig. (2) shows immunostaining for CML, pyrraline, pentosidine, and VEGF in the retinas of nondiabetic SD rats. The prevalence rates of immunoreactivity for CML and VEGF were significantly greater in the

retinas of diabetic rats than in nondiabetic rats ($P < 0.05$, $P < 0.001$, respectively, by the Cochran-Armitage test). There was no significant difference in immunoreactivity for pyrraline and pentosidine between the diabetic and nondiabetic rats ($P = 0.35$, $P = 0.38$, respectively, by Fisher's exact test).

Table 1. Prevalence Rates of Immunostaining for CML, Pyrraline, Pentosidine, and VEGF in the Retinas of Diabetic Rats

	CML	Pyrraline	Pentosidine	VEGF
Minimal	0	5	5	0
Moderate	1	1	1	3
Severe	5	0	0	3

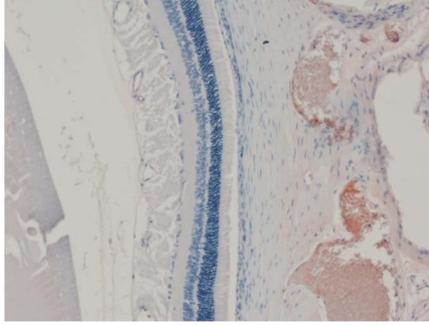
Table 2. Prevalence Rates of Immunostaining for CML, Pyrraline, Pentosidine, and VEGF in the Retinas of Nondiabetic SD Rats

	CML	Pyrraline	Pentosidine	VEGF
Minimal	1	6	10	9
Moderate	7	4	0	1
Severe	2	0	0	0

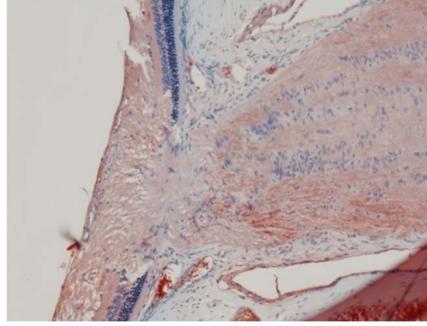
DISCUSSION

The most frequent ocular complications found in diabetes, i.e., cataracts, retinopathy, and neovascular glaucoma, are thought to be caused by increased polyol pathway activity [6-9], activation of PKC [10-14], increased oxidative stress [13,15,16], and accumulation of AGEs as a result of prolonged hyperglycemia. In advanced cases of diabetes, retinal and iris neovascularization may occur, resulting in blindness due to proliferative DR, neovascular glaucoma, or both. VEGF is necessary for neovascularization in diabetic eyes. Increased polyol pathway activity, activation of PKC, oxidative stress, and AGEs could all induce VEGF. Although these factors can be improved with glycemic control, the AGEs are resistant to degradation and continue to accumulate in ocular tissues even in patients with diabetes with good glycemic control. Among the numerous AGEs, CML is thought to be the major one that causes ocular complications in diabetes [13,28,29]. However, pentosidine is more important than CML in the development of diabetic nephropathy [29-31]. Hammes *et al.* reported that accumulation of CML can be identified even in early-stage DR, while in the advanced stages of diabetes, accumulation of CML accompanies progression of DR [18]. Several clinical studies have shown that the level of pentosidine is more related to nephropathy than retinopathy [29,32]. We found that accumulation of CML was observed somewhat in nondiabetic normal SD rats. In SDT rats, staining for CML was stronger than in nondiabetic rats, and the prevalence of VEGF was greater than in nondiabetic rats. However, there was no significant difference in the prevalence of pyrraline and pentosidine between diabetic rats

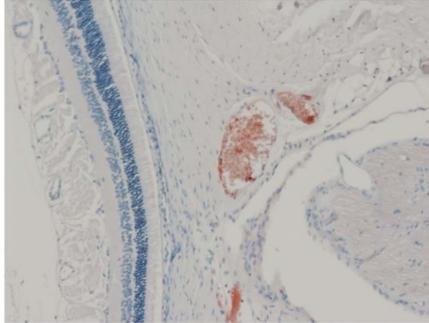
Pyrraline, minimal



CML, severe



Pentosidine, minimal



VEGF, severe

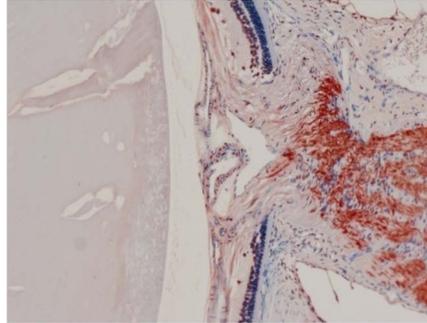
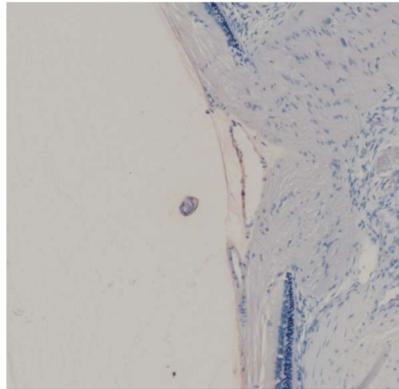
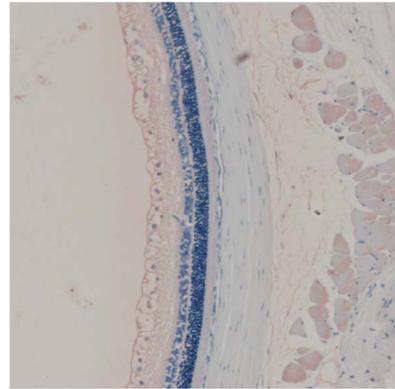


Fig. (1). Immunostaining for AGEs and VEGF in the retinas of diabetic SDT rats. CML and VEGF are clearly seen, but pyrraline and pentosidine are not (original magnification x10).

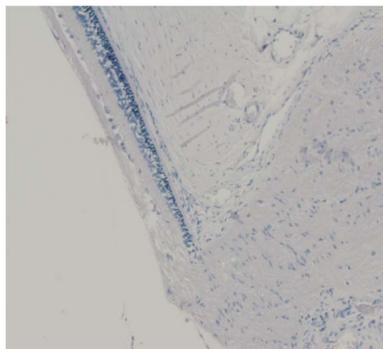
Pyrraline, minimal



CML, moderate



Pentosidine, minimal



VEGF, minimal

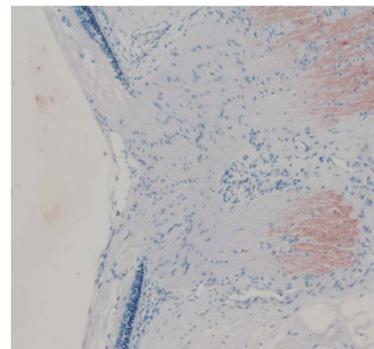


Fig. (2). Immunostaining for AGEs and VEGF in the retinas of nondiabetic SD rats. Staining for CML is weaker than in diabetic SDT rats. Pyrraline, pentosidine, and VEGF are not clearly seen (original magnification x10).

and nondiabetic rats. Therefore, VEGF expression was correlated with CML expression but not pentosidine or pyrraline. It appears that CML may be the most important AGE-inducing neovascularization in the eye, while pyrraline and pentosidine are contributory. Since the relationship of AGEs to diabetic ocular complications in SDT rats closely resembles that in humans, the SDT rat may be a useful animal model for investigating the pathogenesis of diabetic ocular complications.

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